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Effects of Extracellular Calmodulin and Calmodulin Antagonists on B16 Melanoma Cell Growth

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Two drugs known to inhibit the action of calmodulin, prochlorperazine (PCP) and *N*-(6-aminohexyl)-5-chloro-1-napthalene sulfonamide (W7), were investigated for their ability to control cell proliferation in murine B16 melanoma cells in culture. PCP and W7 inhibited [^{3}H]thymidine uptake in these cells, 50% inhibition occurring with 13 μM PCP and 40 μM W7. In the presence of relatively high concentrations of fetal calf serum (FCS), cells withstood high concentrations of both drugs (100 μM PCP and 200 μM W7) and showed increased pigment production. Drug-inhibited DNA synthesis could be reversed by the addition of fresh medium containing FCS or by the addition of exogenous pure calmodulin. Extracellular calmodulin itself stimulated DNA synthesis. FCS was found to contain calmodulin-like activity at concentrations that may be relevant to the stimulation of [^{3}H]thymidine uptake by cells in culture.

There is evidence that the intracellular calcium receptor protein calmodulin plays an important role in cell division; calmodulin has been localized in the formation of the mitotic spindle [1], and the level of calmodulin has been found to double at the G_1/S transition phase of the cell cycle [2,3]. In addition, calmodulin antagonists have been reported to suppress cell division in some cell lines [4,5], and we have reported similar results in normal human lymphocytes [6]. The calmodulin antagonists in use are not specific for the inhibition of calmodulin [7], but Boynton et al [4], in addressing this problem, found that trifluoperazine-inhibited cells could be stimulated by the addition of extracellular calmodulin and that extracellular calmodulin alone stimulated DNA synthesis. More recently, another report of stimulatory effects of extracellular calmodulin on DNA synthesis in normal human lymphocytes has appeared [8].

In malignant cells, mitosis is reported to be less dependent on extracellular calcium than in nonneoplastic cells [9], and calmodulin content is reported to be elevated in several malignant cell lines [10–12].

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Abbreviations:

DMEM: Dulbecco's modified Eagle's medium
EGTA: ethyleneglycoltetraacetic acid
FCS: fetal calf serum
Hepes: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
PBS: phosphate-buffered saline
PCP: prochlorperazine
PCPS: prochlorperazine sulfoxide
TCA: trichloroacetic acid
W7: *N*-(6-aminohexyl)-5-chloro-1-napthalene sulfonamide

We have previously reported the purification of calmodulin from mouse B16 melanoma tumors [13], and in the present study we investigate the use of two calmodulin antagonists, a phenothiazine, prochlorperazine, and *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7), reported to be more specific for the inhibition of calmodulin [5] in the control of cell proliferation in cells cultured from these tumors. In addition, we examine the effect of extracellular calmodulin on DNA synthesis in these cells, and we compare the calmodulin content of these cells with that of other neoplastic and nonneoplastic cells.

MATERIALS AND METHODS

Materials

Cyclic [^{3}H]AMP and [^{3}H]thymidine were obtained from Amersham International Limited, Bucks., U.K. Cyclic AMP, pure pig brain calmodulin, and calmodulin-deficient phosphodiesterase were obtained from Boehringer Mannheim, London. Snake venom (*Ophiophagus hannah*) and Dowex 1-X8 (200–400 mesh) anion exchange resin were obtained from Sigma Chemical Company, Poole, Dorset, U.K.

Medium-199, phosphate-buffered saline (PBS), fetal calf serum (FCS), glutamine, penicillin, and streptomycin were obtained from Gibco-Europe Limited, Paisley, U.K. All tissue culture plastics were obtained from Sterilin, Teddington, Middlesex, U.K. All other chemicals were of analytical grade.

Prochlorperazine (PCP) and prochlorperazine sulfoxide (PCPS) were gifts from Smith, Kline and French Labs., Limited, Welwyn Garden City, Herts., U.K. W7 was a gift from Dr. M. Blackburn, Sheffield University.

Calmodulin Assay

Heat-treated extracts of confluent cells were prepared as described previously [14]. Essentially, tissue culture medium was removed and cells rinsed with 0.9% (w/v) NaCl and buffer (40 mM Tris-HCl, pH 7.4, at room temperature, 0.1 mM $CaCl_2$, 50 mg/l phenylmethylsulfonylfluoride, and 50 μl/l pepstatin A) in the ratio of 1 ml per 9-cm^2 culture dish added. Cells were detached from dishes by scraping and homogenized using a glass Dounce homogenizer. A sample of homogenate was retained for protein measurement, (assay method as in [15]), and the remainder was heated to 90°C for 5 min. Denatured protein was removed by centrifugation at 10,000×g for 10 min, and supernatants were stored at −20°C until assay.

Calmodulin activity in these extracts was measured by the ability of cell extracts to activate a calmodulin-dependent cyclic nucleotide phosphodiesterase, as used previously [6]. Optimal assay conditions, using a beef heart calmodulin phosphodiesterase obtained from Boehringer Mannheim, London, were as follows: Assays contained in a final reaction mixture of 400 μl, 40 mM Tris-HCl, pH 7.0, at 37°C, 4 mM 2-mercaptoethanol, 5 mM $MgCl_2$, [^{3}H]cyclic AMP (2×10^5 cpm/tube), 100 μM cyclic AMP, 25 μM $CaCl_2$, and calmodulin or heat-treated cell extract as required. Incubations of 15 min at 37°C were commenced by the addition of enzyme (approximately 1.5 mU/tube) and terminated by transferring tubes to a boiling water bath. Tubes were then transferred to ice. The 5'-AMP formed during this incubation with phosphodiesterase is then converted to adenosine by the 5'-nucleotidase present in snake venom. Then 100 μl snake venom in distilled water was added to the tubes and incubated for 10 min at 37°C before returning the tubes to ice. Product nucleosides were separated from unreacted substrate using Dowex anion exchange resin. Dowex slurry (1 ml; 1 part Dowex to 4 parts methanol) was added to each tube, and the contents were mixed and centrifuged at 1000g for 10 min. Then

supernatant, containing adenosine, was decanted from each tube and counted for tritiated adenosine using standard liquid scintillation counting techniques. Calmodulin activity in cell extracts and in heat-treated extracts of FCS was determined at 3 dilutions for each extract, comparing the activity present with that produced by a pure calmodulin standard. This activity was then related to the amount of protein in the original cell homogenate and the results expressed as milligrams of calmodulin activity per milligram of cell protein.

[^{3}H]Thymidine Uptake into B16 Melanoma

The B16 mouse melanoma was serially transplanted in C57BL mice. Primary cultures were derived from the tumors and grown in a CO_2 incubator in Medium-199 with Hanks' salts and 20 mM Hepes buffer supplemented with 10% FCS and containing penicillin (50 IU/ml) and streptomycin (50 μg/ml).

For [^{3}H]thymidine uptake, cells were grown in multiwell plates (6 wells of 3.5 cm diameter) at a seeding density of 1–2 × 10^5 cells per milliliter until cells covered approximately 25% of the plates (as determined by eye; this usually took 2–3 days). Cells were then washed with PBS and incubations commenced by the addition of 1 ml of medium per well containing 1.5 μCi [^{3}H]thymidine and calmodulin or calmodulin antagonists as required. Incubations were terminated at times described by removal of medium, and cells were washed with PBS followed by 400 μl of 10% TCA for 10 min at 4°C and then finally digested in 400 μl of 1 N NaOH for 16 h at 37°C. Radioactivity in the cell digests was then measured. Incubations were performed in triplicate, and each experiment was repeated on a minimum of 3 occasions.

Measurement of Pigmentation

An indication of changes in the amount of pigment present in melanoma cultures was obtained by analyzing photographs of confluent cultures with an epidiascope attached to a Quantamat 720 image-analyzing computer (Cambridge Instruments Ltd.) interfaced with a Hewlett-Packard 9815A desktop calculator. For this study, cells were grown to confluence in 25-cm^2 Falcon flasks in Medium-199 with Hanks' salts and 20 mM Hepes buffer supplemented with 20% FCS and containing penicillin (50 IU/ml) and streptomycin (50 μg/ml).

Other Cell Lines

In addition to B16 melanoma cells, primary cultures of cells were also obtained from human articular cartilage, human normal bone, human nontoxic thyroid goiter, human prolactinoma (using material obtained from surgery), pig thyroid (using freshly slaughtered animals), and osteogenic sarcoma tumors serially transplanted in rats. A cloned cell line, rat GH_3 cells, derived from a rat pituitary tumor was also used. Cells were maintained in Ham's FIO medium (GH_3), Medium-199 (pig and human thyroid), or DMEM (all others).

RESULTS

The amount of biologically active calmodulin present in cultured B16 melanoma cells was compared with that present in other neoplastic and nonneoplastic cells in the laboratory. All cells were compared at confluence. As can be seen from Table I, calmodulin levels in B16 melanoma cells were similar to those found for several nonneoplastic cells at confluence.

Two calmodulin antagonists were examined for their effects on cell division, a common phenothiazine, PCP, together with its relatively inactive calmodulin antagonist analogue, PCPS, and a drug reported to be more specific for calmodulin inhibition, W7.

TABLE I. *Calmodulin content in cultured cells*

Cell	Calmodulin (μg/mg protein, $\bar{x} \pm$ SD, $n = 3$)[a]
Rat pituitary GH3 cells	0.66 ± 0.03
Mouse B16 melanoma cells[b]	0.70 ± 0.04
Human articular cartilage chondrocytes	0.79 ± 0.07
Pig thyroid cells (normal)	0.87 ± 0.03
Human thyroid cells (nontoxic goiter)	0.99 ± 0.11
Human normal bone cells	1.22 ± 0.11
Rat osteogenic sarcoma cells[b]	1.48 ± 0.21
Human prolactinoma cells[b]	2.30 ± 0.05

[a] Calmodulin activity was determined in confluent cultures of cells as described in *Materials and Methods*.

[b] Neoplastic cells.

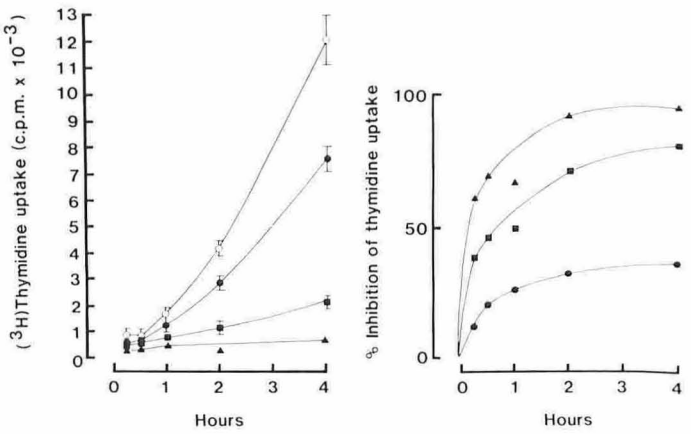

FIG 1. Time course of inhibitory effect of W7 on [^{3}H]thymidine uptake in B16 melanoma cells. *Left*, [^{3}H]Thymidine uptake during 15 min, 30 min, 1 h, 2 h, or 4 h incubation with [^{3}H]thymidine (1.5 μCi/ml) in the absence of drug (○) and in the presence of 50 μM W7 (●), 100 μM W7 (■), or 200 μM W7 (▲). Results shown are means ± SE of triplicate cultures of a single experiment. *Right*, The data shown on the left is used to calculate the degree of inhibition of [^{3}H]thymidine uptake produced by 50 μM W7 (●), 100 μM W7 (■), and 200 μM W7 (▲) at each time point.

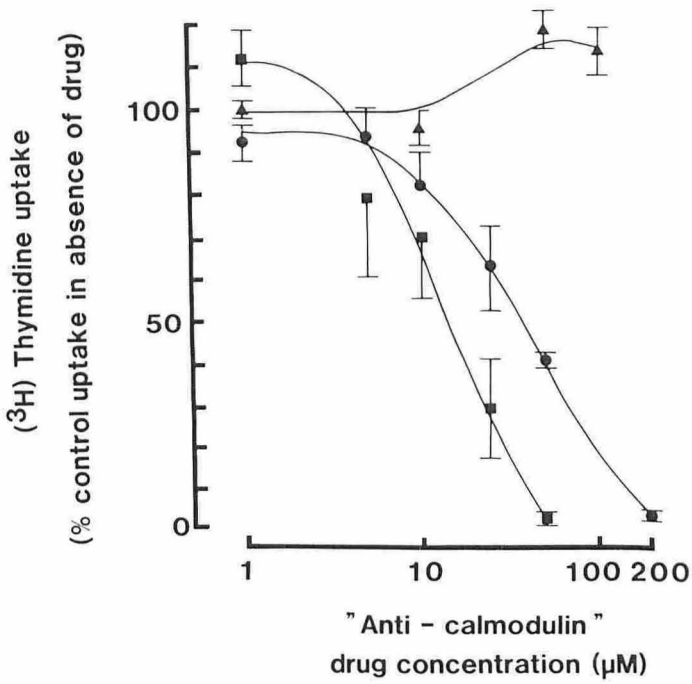

FIG 2. Effect of PCP (■), PCPS (▲), and W7 (●) on [^{3}H]thymidine uptake in B16 melanoma cells over 48 h. Results are means ± SE of 3 experiments with PCP and PCPS and 4 experiments with W7. Control values for [^{3}H]thymidine uptake, which ranged from 2 × 10^3 to 5 × 10^3 cpm, are designated 100% to allow experiments to be combined.

The inhibition of DNA synthesis developed during the first 2 h of exposure to the drugs as is shown in Fig 1 for the drug W7. During a more prolonged period of exposure to the drugs (48 h), this inhibition was maintained. Over this period (Fig 2), [^{3}H]thymidine uptake was reduced 50% by 40.5 ± 5.8 μM W7 ($\bar{x} \pm$ SE, $n = 4$) and 13.0 ± 3.9 μM PCP ($\bar{x} \pm$ SE, $n = 3$). PCPS was without effect on [^{3}H]thymidine uptake up to 100 μM.

In addition to the inhibitory effect of the drugs on DNA synthesis, it was clear that under the experimental conditions described, higher concentrations of these drugs also caused cell detachment. This was apparent by eye and was also obvious in

the final protein content of the wells, which was less than that present at the beginning of incubations. This effect could be clearly separated in terms of concentration dependence from the effect on DNA synthesis. In the experiments shown in Fig 2 (using 10% FCS), a 50% reduction in the amount of cell protein remaining in the wells at the end of 48 h incubation occurred with 120 ± 24 μM W7 ($\bar{x}$ ± SE, $n = 4$) and 36.7 ± 7.2 μM PCP ($\bar{x}$ ± SE, $n = 3$). PCPS had no effect on cell detachment. In the absence of FCS, cell detachment occurred rapidly, e.g., in one such experiment a 50% loss of attached cells occurred with 40 μM W7 and 30 μM PCP within 15 min. However, in the presence of 20% FCS, confluent cultures of melanoma cells maintained in concentrations of W7 or PCP sufficient to completely inhibit cell division (200 μM W7 or 100 μM PCP) showed minimal cell detachment and continued to produce pigment. This increase in pigmentation was evident by eye in confluent cultures of B16 melanoma after 4 to 5 days of exposure to these drugs and was verified by the use of an image-analyzing computer. Photographs of cultures exposed to 200 μM W7 for 5 days were an average of 145% darker than photographs of control cultures. Photographs of cultures exposed to 100 μM PCP for 5 days were 93% darker than control cultures.

Inhibition of DNA synthesis produced by the drugs could be reversed either by replacement of medium or by addition of extracellular pure calmodulin to the medium. Figure 3 shows that cells exposed to 50 μM W7 for 18 h showed a significant increase in [^{3}H]thymidine uptake in response to the subsequent addition of 1 μg/ml of pure calmodulin to the medium. (Similar results have also been obtained using cells exposed to W7 for 2

TABLE II. *Effect of extracellular calmodulin on [^{3}H]thymidine uptake*

Experiment[b]	[^{3}H]Thymidine uptake (cpm × 10^{-3} per 48 h)[a]		
	Control	+ Calmodulin (1 μg/ml)	Effect
1	6.34 ± 0.32	9.44 ± 0.54	+49%
2	9.10 ± 0.9	15.0 ± 1.4	+65%
3	9.3 ± 0.6	20.9 ± 0.8	+125%
4	9.5 ± 1.5	16.6 ± 0.2	+75%
5	13.8 ± 1.6	14.5 ± 2.5	N.S.
6	26.3 ± 3.5	27.5 ± 2.6	N.S.
7	35.0 ± 1.3	36.0 ± 1.2	N.S.
8	45.3 ± 4.3	47.1 ± 3.7	N.S.

[a] [^{3}H]Thymidine uptake in cultures was as described in *Materials and Methods.*

[b] Experiments are arranged in ascending order of [^{3}H]thymidine uptake rather than in order of execution.

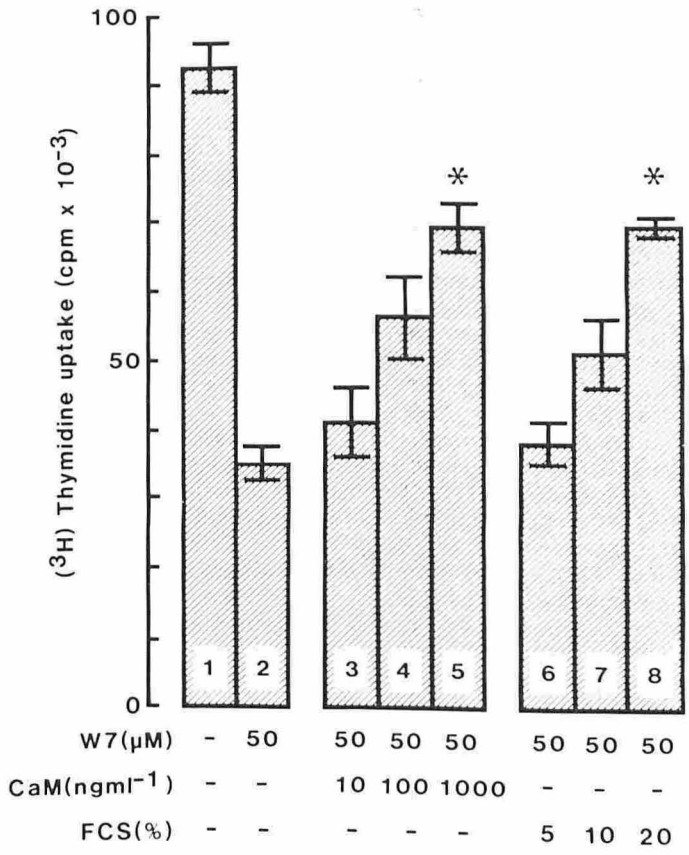

FIG 3. Effect of extracellular calmodulin and of medium replacement on [^{3}H]thymidine uptake in W7-inhibited cells. Results shown are means ± SE of triplicate cultures of a single representative experiment. Histograms 1–8 represent results of 1-ml cultures pretreated with either no drug (control, histogram 1) or 50 μM (histograms 2–8). All cultures were then incubated with [^{3}H]thymidine for 7 h, during which period calmodulin was added to cultures represented by histograms 3–5 (10, 100, and 1000 ng/ml, respectively) and medium was changed in cultures 6–8 for medium containing 5, 10, or 20% FCS, respectively. Asterisk indicates a significant increase ($p < 0.05$) in [^{3}H] thymidine uptake compared with uptake in the presence of 50 μM W7 alone.

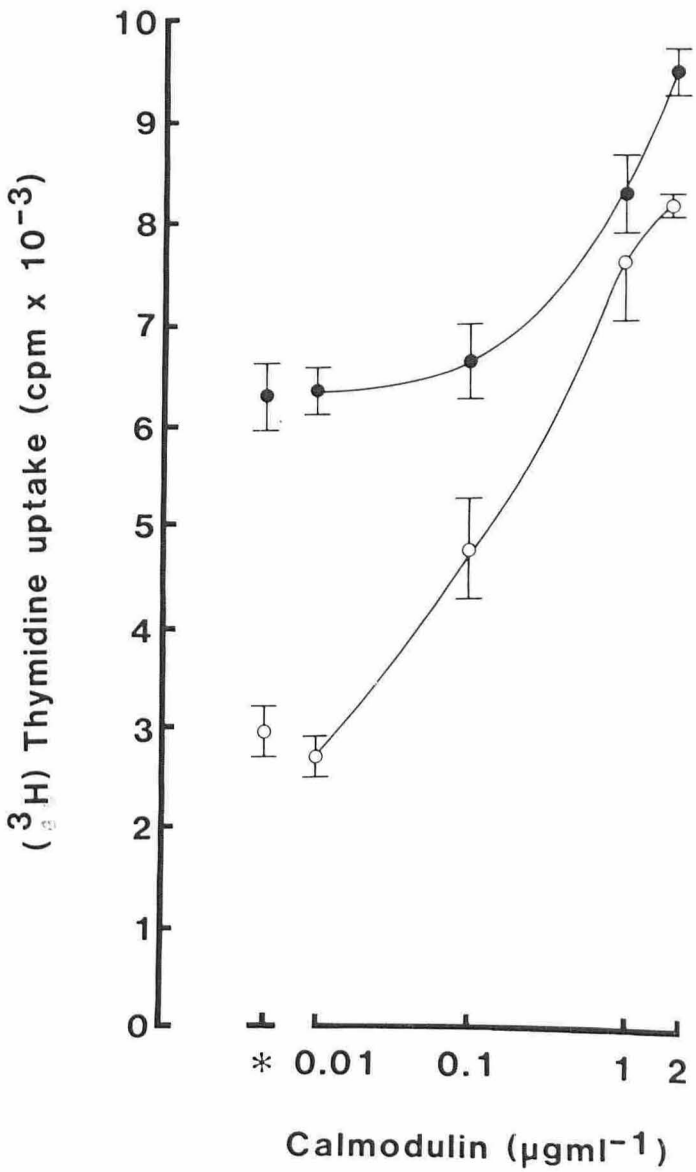

FIG 4. Stimulatory effect of extracellular pure calmodulin on [^{3}H] thymidine uptake over 48 h in B16 melanoma cultures containing either 10% (●) or 5% (○) FCS. Results shown are means ± SE of triplicate cultures of a single experiment. Values indicated by asterisk represent [^{3}H]thymidine uptake in the absence of calmodulin.

or 4 h prior to calmodulin addition.) This concentration of calmodulin represents 5.98×10^{-11} mol calmodulin per 1 ml incubation, compared with the 5×10^{-8} mol W7 already present in the incubation. In the experiment shown in Fig 3, [^{3}H] thymidine uptake was measured over 7 h of exposure to calmodulin. The stimulatory response to calmodulin could be first detected between 1 and 2 h of exposure to calmodulin. DNA synthesis in W7-inhibited cells could also be reversed by the replacement of medium containing FCS, as shown in Fig 3, with cells retaining the ability to respond to increasing concentrations of FCS.

In an investigation of the effects of extracellular calmodulin on DNA synthesis in the absence of calmodulin antagonists, we found that the response of the melanoma cells to extracellular calmodulin varied and that this response appeared to be affected by the concentration of FCS present. In 8 consecutive experiments in the presence of 10% FCS, the addition of pure calmodulin increased [^{3}H]thymidine uptake in 4 experiments, as shown in Table II. Calmodulin was effective in those experiments having the lowest basal rate of [^{3}H]thymidine incorporation. Since we knew that growth in B16 melanoma cells in culture is highly dependent on the concentration of FCS present (maximally stimulating increase in [^{3}H]thymidine uptake produced by 25% FCS), we reduced FCS to 5% in several experiments to see if this would facilitate the study of the effects of extracellular calmodulin on [^{3}H]thymidine uptake in these cells. As shown in Fig 4, the stimulatory effects of extracellular calmodulin can be seen more clearly in the presence of 5% rather than 10% FCS. As little as 100 ng/ml calmodulin (5.98×10^{-9} M) produced significant stimulation in the presence of 5% FCS. We subsequently examined FCS for the presence of calmodulin-like activity and found calmodulin-like activity in 6 different batches of FCS ranging from 0.9 to 8 μg/ml (3.8 ± 1.0 μg/ml, $\bar{x} \pm$ SE, $n = 6$) determined by the ability of heat-treated extracts of these sera to activate a calmodulin-dependent phosphodiesterase. This activity diluted in parallel to a pure calmodulin standard and was inhibited by the addition of either EGTA (100 μM) or W7 (100 μM).

DISCUSSION

We found calmodulin content in B16 melanoma cells at confluence to be within the range found for nonneoplastic confluent cells in our laboratory (0.66 to 1.22 μg calmodulin per milligram of protein). However, increased calmodulin content has been reported in other neoplastic cells [10–12]. In the present study, neoplastic cells derived from a rat osteosarcoma and from a human prolactinoma showed calmodulin levels 64% and 155% greater than the calmodulin levels (0.90 ± 0.08 μg calmodulin per milligram of protein, $\bar{x} \pm$ SE) determined in 5 nonneoplastic cell types.

DNA synthesis in B16 melanoma cells was inhibited by PCP and W7. The gradual development of inhibition over the first 2 h of exposure to the drugs is probably explained by the use of nonsynchronized populations of cells in this study, since Boynton et al [4] found that only 15 min of preexposure to trifluoperazine was sufficient to block the normal DNA-synthetic response to calcium in rat liver cells. B16 melanoma cells grown in the continued presence of the drugs for 48 h did not escape from this inhibition, although the inhibition was reversed by the removal of drugs. In terms of potency, we found that slightly higher concentrations of W7 (40.5 ± 5.8 μM, $\bar{x} \pm$ SE) and PCP (13.0 ± 3.9 μM, $\bar{x} \pm$ SE) are required for half-maximal inhibition of thymidine uptake in B16 melanoma cells than are required for the inhibition of calmodulin-dependent phosphodiesterase activity [17]. These 50% inhibition values were determined in the presence of 10% FCS. Since subsequent experiments revealed FCS to contain calmodulin-like activity, it is possible that the calmodulin antagonists may appear more potent in the absence of serum.

With both PCP and W7 we observed cell detachment. Hidaka et al [5], using 5% FCS, found that W7 detached cells from the plate at concentrations greater than 60 μM. We found that the presence of higher concentrations of FCS (20%) appeared to give some measure of protection to the cells against this effect of the drugs on cell attachment. In addition, cells exposed to high concentrations of W7 and PCP in the presence of 20% FCS continued to produce pigment, having increased pigment production compared with control cultures. This suggests that the inhibitory action of the drugs on DNA synthesis is not simply due to a nonspecifically damaging effect of the drugs on the cells and is further supported by the finding that drug-inhibited [^{3}H]thymidine uptake could be restimulated by the subsequent addition of fresh medium containing FCS or, as in the study of Boynton et al [4], by the subsequent addition of pure calmodulin. It should be emphasized that the pure calmodulin added was not present in sufficient concentration to itself inactivate the calmodulin antagonist drugs present in the culture medium.

The effects of extracellular calmodulin on DNA synthesis were first described by Boynton et al [4] for rat liver cells, and more recently, exogenous calmodulin has been reported to stimulate lymphocyte proliferation in humans [8]. The stimulatory effects of extracellular calmodulin on thromboxane production in human platelets have also been reported [18]. The mode of action of extracellular calmodulin is unknown. In our study we found significant stimulation could be observed at 6×10^{-9} M (100 ng/ml) if FCS were lowered to 5%. Medium containing FCS could also restimulate calmodulin antagonist-inhibited cell division. In the studies of Boynton et al [4] and Gorbacherskaya et al [8], higher concentrations of calmodulin were required for significant stimulation of cell growth (10^{-7} M and 10^{-6} M, respectively). Calmodulin, although present in all eukaryote tissues, has not so far been described in extracellular fluid to the best of our knowledge. Our finding of calmodulin-like activity in FCS is therefore surprising. Since we observed the stimulatory effects of pure calmodulin on cell proliferation at levels equivalent to the concentration of "calmodulin-like" activity in 10% FCS, our results suggest that the presence of this activity in FCS may merit further investigation.

Our preliminary work (unpublished) suggests that the source of calmodulin-like activity in FCS is the platelet. However, the growth promoting effects of FCS in this study may be due to the presence of any of the many well-documented growth factors known to be present in FCS.

In summary, the purpose of the present study was to investigate whether calmodulin antagonists could be of use in inhibiting the growth of B16 melanoma cells in culture. We conclude that these drugs do inhibit [^{3}H]thymidine uptake in these cells, that the effects of these drugs are persistent, and that their effects are not due to nonspecific damage to the cells. During the preparation of this manuscript, two reports of the use of calmodulin antagonists in the treatment of growth in solid tumors in animals have appeared [19,20]. The results of these studies, which include B16 melanoma tumors in mice [20], show that these drugs are effective in controlling cell proliferation in neoplastic tissue in animals.

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